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Quantitative study of the development of the optic nerve in rats reared in the dark during early postnatal life*

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INTRODUCTION

Several investigators have shown the importance of visual experience to the proper development of the mammalian visual system. Rearing animals in total darkness during a critical period in early development has been reported to cause long lasting changes in various components of the visual pathway. Such effects have been described for the various components of the visual system of several animals including cats, rats, mice and monkeys (Wendell-Smith, 1964; Gyllensten, Malmfors & Norrlin-Gretive, 1966; Gyllensten & Malmfors, 1963; Coleman & Riesen, 1968; O'Kusky, 1985; Ward & Tremblay, 1981; Cragg, 1969; Gyllensten *et al.* 1965; Lund & Lund, 1972; Fifkova & Hassler, 1969; Mackay & Bedi, 1987; Borges & Berry, 1978; Gyllensten, 1959; Cragg, 1967; Prada, 1987; Bedi, 1989; MacAvoy, Salinger & Garraghty, 1990).

Surprising though it may seem, relatively few of these studies have been concerned with the effects of light deprivation on the fibre composition of the optic nerves. There are some studies on this aspect published by Gyllensten & Malmfors (1963) and Gyllensten *et al.* (1966). These researchers examined the effects of rearing mice in total darkness for a period of two to four months immediately after birth. In other experiments some rats were deprived of light for a period of three months in early adult life (i.e. between four and seven months of age.) They found that mice reared in darkness during early postnatal life showed no significant differences in the fibre composition of the optic nerve compared to controls. In adult mice, lack of visual stimulation caused an increase in the number and diameter of the optic nerve fibres.

These early quantitative studies of the composition of the optic nerve were carried out using the relatively low magnifications and resolutions available with phase contrast light microscopical techniques. Subsequent studies (e.g. Forrester & Peters, 1967) have indicated that many of the small diameter myelinated and non-myelinated fibres cannot be properly resolved with these light microscopical methods. This indicates the need for a re-examination of the effects of dark-rearing during early postnatal life on the fibre composition of the optic nerves using the high resolutions available with electron microscopical techniques. We report the results of such an investigation in this paper.

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MATERIALS AND METHODS

Animals and conditions

Pregnant black and white hooded Lister rats were placed in separate cages which were kept in a 'dark' room. This room was maintained in total darkness throughout the day apart from a few minutes each day when it was illuminated by a 'dim' red light in order to check the well-being of the animals. Care was taken to ensure no extraneous light was admitted into the room, even when entry was gained. The day of birth was designated Day 0 of the pup's life. On Day one, litters were standardised to contain between 8 and 10 pups at least two of which were female. The litters were then randomly assigned to light or dark conditions. Those assigned to the dark conditions were kept in the 'dark' room described above. Those to be raised in the light conditions were placed in a second room, which was maintained on a 12 hours light/12 hours red cycle. At 20 days of age, male pups were weaned by separating them from their mothers. Pups were housed two to a cage and kept in the previously designated lighting conditions. The female pups were discarded at this stage.

At 30 days of age a group of dark-reared and light-reared rats was killed for histological examination (see below). The remaining rats were all placed in the light room irrespective of their previously designated lighting conditions. Groups of these rats were killed at 65 days of age. Food and water was available to all rats *ad libitum* throughout the experiment.

Tissue preparation

Rats were anaesthetised with sodium pentobarbitone (Sagatal, May & Baker, UK) and perfused via the left ventricle with about 450 ml of 2·5% glutaraldehyde solution in 0·1 M phosphate buffer (pH 7·3). The dark-reared rats were anaesthetised in the dark room before being removed to an illuminated laboratory for the perfusion procedure. An hour after perfusion, the brains were dissected out. From each brain a 2 mm length of optic nerve, situated approximately midway between the posterior pole of the eyeball and the optic chiasma, was obtained and immersed in fresh fixative and left overnight. The tissue was then washed in several changes of buffer, postfixed in 0·5% osmium tetroxide for three hours, dehydrated in a series of ethanol solutions and embedded in Epon.

Sampling procedures

Transverse semithin sections (about 0·5 μm) and adjacent ultrathin sections (about 70 nm) were cut from each optic nerve on an LKB ultramicrotome. The semithin sections were picked up on glass slides, stained with toluidine blue and photographed using a Nikon Light microscope. The micrographs were printed to a final magnification of about $\times$ 200. A photomicrograph of a stage micrometer was included in each film and taken as a magnification standard. These photomicrographs were used to measure the cross-sectional area of each nerve using an MOP image analyser (Kontron, Messgerate, GFR). These measurements were later used to estimate the total number of nerve fibres in each optic nerve.

The same blocks of tissue were subsequently sectioned for electron microscopy. An ultrathin section was cut from each nerve, picked up on a 300-mesh grid and stained with uranyl acetate and lead citrate. Each section was examined with a Hitachi H-500 electron microscope operated at an accelerating voltage of 75 kV.

A systematic random sampling procedure (Weibel, 1979) was employed to obtain a sample of electron micrographs from a single section through each nerve. This entailed

taking a micrograph in a predetermined corner of each grid square containing nerve tissue. The sampling strategy adopted ensured that at least 20 electron micrographs were obtained from each optic nerve examined. Electron micrographs were taken at a nominal magnification of 10000 and printed to a final magnification of about 38000. A micrograph of a cross grating replica having 2160 lines per millimetre was used as a magnification calibration standard.

Transverse sections through non-myelinated fibres and glial cell processes can be difficult to distinguish from each other unless relatively high magnifications are used, as was the case in the present experiment. At these relatively high magnifications axons can be distinguished from glial cell processes as they contain both neurofilaments and neurotubules whereas glial cells have a darker-staining fibrillar cytoplasm and are often closely associated with myelin sheaths (Peters, 1964).

Estimation of minimum fibre diameter

The 'minimum axonal diameter' (Sima & Sourander, 1974) was measured for each axon which appeared within the counting frame and which did not touch the 'forbidden line' (Gundersen, 1977). The 'minimum axonal diameter' was taken as the minor axis of any given axon. In the case of myelinated axons, the 'minimum fibre diameter' (axon + myelin sheath) and the thickness of the myelin sheath were measured in addition to the 'minimum axonal diameter'. These measurements were also made with the aid of an MOP image analyser.

Estimation of fibre number

A rectangular frame, slightly smaller than the print area of each micrograph, was placed in turn over each electron micrograph of optic nerve tissue. Myelinated and non-myelinated axons were counted separately. The 'forbidden' line rule (Gundersen, 1977) was used to determine whether or not to include any given nerve fibre profile within the counts. The myelinated and non-myelinated fibre densities for each rat were calculated by relating these counts to the total area of tissue sampled (rectangular frame area in absolute units $\times$ number of micrographs analysed). These values, together with the cross-sectional area of the entire optic nerve (as determined above), were used to estimate values for the total number of myelinated and non-myelinated optic nerve fibres in each animal.

Statistics

All measurements and estimates were first made for each individual rat. These were later pooled for any given group of animals to yield mean $\pm$ S.E. Differences between groups were tested using Student's t test and analysis of variance (ANOVA) procedures (Sokal & Rohlf, 1981).

RESULTS

Figures 1 and 2 show light and electron micrographs of transverse sections through a control rat optic nerve. Such micrographs were used to estimate the total number of both myelinated and non-myelinated optic nerve fibres, as well as the mean minimal axonal and fibre diameters and mean thickness of the myelin sheath. There were no obvious qualitative differences between dark- and light-reared rats in the general appearance of the optic nerve tissue as seen in the micrographs obtained for these counting and measuring procedures.

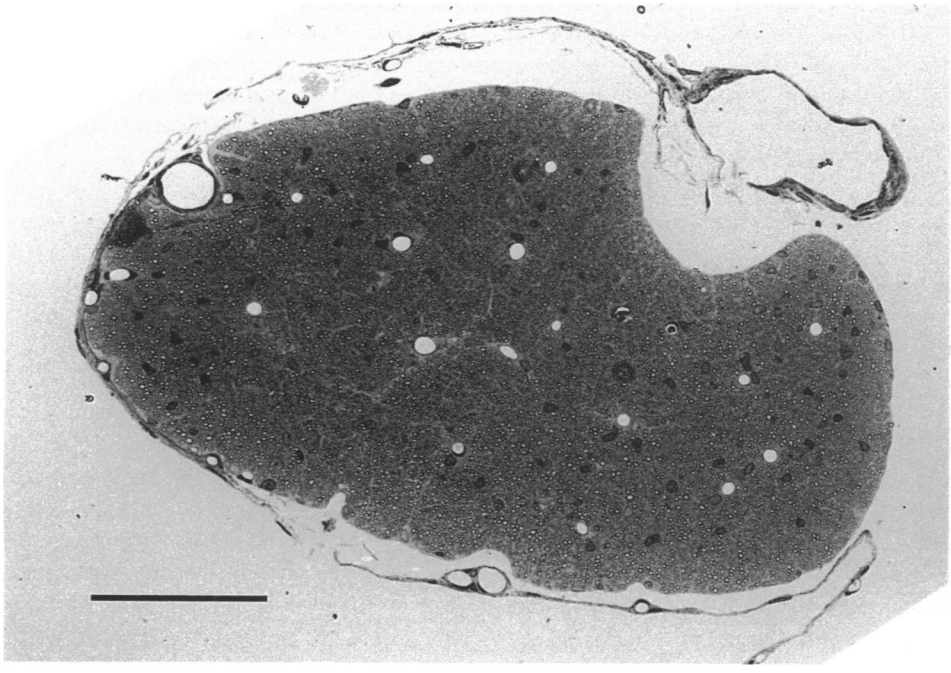

Fig. 1. A light micrograph of a 0·5 μm thick cross-section of the optic nerve of a 30 days old light-reared black and white hooded Lister rat. Bar, 100 μm.

Table 1. *Estimates of fascicular cross-sectional area of the optic nerves of light- and dark-reared rats*

	Age (days)	Light (n, 8)	Dark (n, 8)
Fascicular cross-sectional area (mm^2)	30	0·11 ± 0·007	0·12 ± 0·002
	65	0·17 ± 0·006	0·17 ± 0·003

Results are given as mean ± S.E.
n, number of rats.

Cross-sectional areas of optic nerves

In the region of optic nerve sampled the mean transverse fascicular cross-sectional area was about 0·11 mm^2 in both 30 days old light- and dark-reared rats (Table 1). Two-way ANOVA tests revealed a significant effect of age but no significant effect of lighting conditions, nor interaction (Table 5).

Axonal and fibre diameters

Myelinated axons

Figure 3 gives the frequency distribution histograms of the myelinated optic nerve fibre (i.e. axon + myelin sheath) diameters in light- and dark-reared rats. At 30 days of age (Fig. 3*a*) the dark-reared animals showed fewer small fibres in size Class 2 and more fibres in size Class 4 than were seen in age-matched controls. This resulted in the dark-reared rats having a signifcantly larger mean minimum fibre diameter than light-

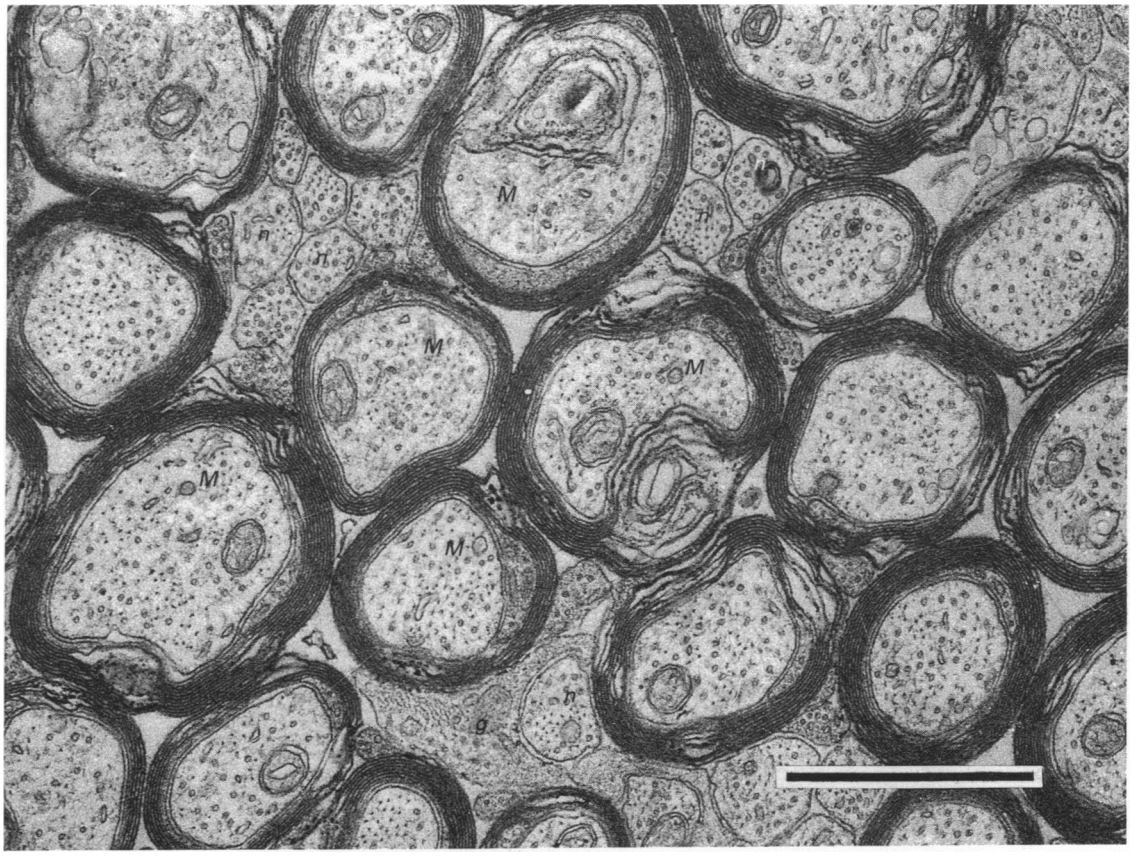

Fig. 2. An electron micrograph of a portion of a rat optic nerve from a 30 days old light-reared rat. *M*, myelinated fibre; *n*, non-myelinated fibre; *g*, glial cell process. Bar, 1 μm.

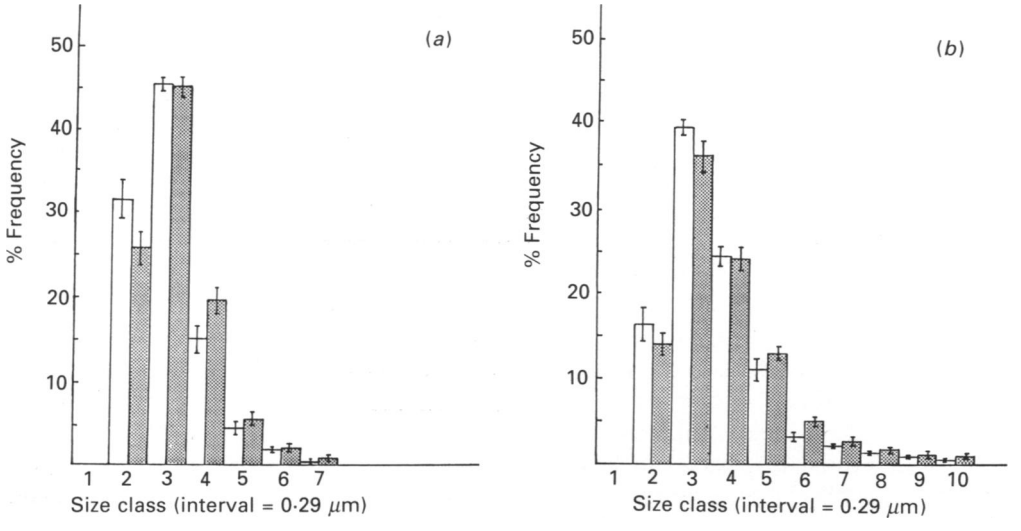

Fig. 3(*a–b*). Size (diameter) frequency distributions of myelinated fibres in control ('open' bars) and dark-reared ('stippled' bars) rats. The bars and vertical lines represent means ± standard errors. (*a*) 30 days old rats; (*b*) 65 days old rats.

Table 2. *Mean ± S.E. myelinated fibre (axon) diameter*

	Age (days)	Light (n, 8)	Dark (n, 8)
Myelinated fibre diameter (μm)	30	0·73 ± 0·015	0·79 ± 0·012**
	65	0·93 ± 0·020	0·99 ± 0·026
Myelinated axon diameter (μm)	30	0·46 ± 0·013	0·51 ± 0·011*
	65	0·57 ± 0·015	0·63 ± 0·021*
Thickness of myelin sheath (μm)	30	0·11 ± 0·003	0·11 ± 0·004
	65	0·13 ± 0·003	0·14 ± 0·004

Results are given as mean ± S.E.; $*P < 0·05$; $**P < 0·01$. n, number of rats.

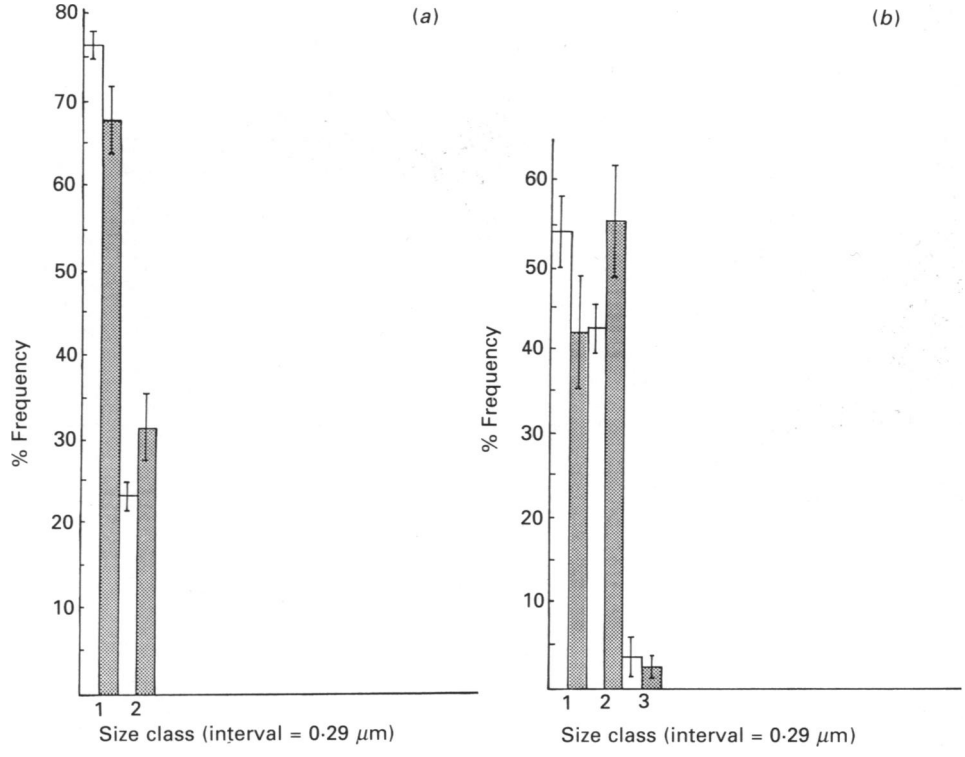

Fig. 4(*a–b*). Size (diameter) frequency distributions of non-myelinated fibres in control ('open' bars) and dark-reared ('stippled' bars) rats. The bars and vertical lines represent means ± standard errors. (*a*) 30 days old rats; (*b*) 65 days old rats.

reared controls at this age (Table 2). This effect did not seem to be influenced by the period of rehabilitation in more normal lighting conditions. Thus, at 65 days of age the dark-reared rats also showed fewer fibres in size Classes 2 and 3 and more in size Classes 5 and 6 (Fig. 3*b*). It was noted that the mean minimum fibre diameter increased significantly between 30 and 65 days of age in both control and experimental animals (Table 2). A two-way ANOVA test of these data showed statistically significant effects of lighting conditions and age but no significant interaction between them (Table 5).

Further analysis revealed that the larger mean fibre diameters observed in the dark-

Table 3. *Mean ± S.E. non-myelinated fibre diameter*

	Age (days)	Light (n, 8)	Dark (n, 8)
Non-myelinated fibre diameter (μm)	30	0·25 ± 0·004	0·27 ± 0·008*
	65	0·31 ± 0·012	0·32 ± 0·010

Results are given as mean ± S.E.; $*P < 0·05$.
n, number of rats.

Table 4. *Estimates of myelinated and non-myelinated axon number in the optic nerves of light- and dark-reared rats*

	Age (days)	Light (n, 8)	Dark (n, 8)
Total fibre number (myelinated-non-myelinated)	30	139210 ± 11311	134187 ± 6475
	65	120252 ± 7151	110708 ± 4185
No. of myelinated fibres (percentage)	30	98226 ± 8312 (70·5%)	101237 ± 4695 (75·6%)
	65	107938 ± 6746 (89·7%)	103119 ± 3807 (93·2%)
No. of non-myelinated fibres (percentage)	30	40984 ± 5043 (29·5%)	32949 ± 2831 (24·4%)
	65	12314 ± 869 (10·3%)	7589 ± 1047* (6·8%)

Results are given as mean ± S.E.; $*P < 0·01$.
n, number of rats.

reared rats were due to larger diameter axons rather than increased myelin sheath thickness at both 30 and 65 days of age (Table 2).

Non-myelinated axons

Figures 4(*a*) and 4(*b*) give the size frequency distributions of the non-myelinated axon diameters in light- and dark-reared rats at 30 and 65 days of age respectively. Dark-reared rats showed fewer fibres in size Class 1 and more fibres in size Class 2 than light-reared controls in both age groups examined. This resulted in a small but significant difference in the mean minimum axon diameter between dark- and light-reared rats at 30 days of age, but not at 65 days of age (Table 3). Once again there was a significant increase in the axon diameter with increasing age (Table 3). Two-way ANOVA of these data showed a significant effect of age but not lighting condition (Table 5). The interaction term was also not statistically significant (Table 5).

Total nerve fibre number

Thirty days old control and dark-reared rats both had an average of about 135000 fibres per optic nerve (Table 4). Of these, about 70%–75% were myelinated fibres, the remainder being non-myelinated. By 65 days of age the total number of fibres had fallen to between 110000 and 120000 per optic nerve. However the proportion of these fibres which were myelinated had increased to about 90%–93% (Table 4). The total number of non-myelinated fibres was significantly smaller in 65 days old previously dark-reared rats compared with age-matched controls (Table 4). Two-way ANOVA

Table 5. *Results of the two-way ANOVA tests on the various measurements of optic nerve from 30 and 65 days old dark- and light-reared rats*

Source of variation	F value[1]		
	Light/Dark	Age	Interaction
Cross-sectioned area	2·58	133·90**	0·11
Total fibre number	0·89	7·55*	0·09
No. of myelinated fibres	0·02	0·89	0·41
No. of non-myelinated fibres	4·61*	82·71**	0·31
Myelinated fibre diameter	9·71**	107·42**	0·02
Myelinated axon diameter	13·04**	58·22**	0·07
Thickness of myelin sheath	2·66	75·21**	1·33
Non-myelinated fibre diameter	3·86	44·90**	0·23

[1] Degree of freedom, 1,28.
* $P < 0·05$; ** $P < 0·01$.

tests on these data revealed a significant effect of age but no significant effect of lighting conditions, nor any significant interaction between age and lighting conditions (Table 5).

DISCUSSION

Our results show that rearing rats in total darkness for a period of 30 days in early postnatal life does not significantly affect the total number of optic nerve axons. However the proportion of these fibres that are myelinated increases slightly in the dark-reared rats. This effect is seen even following a short period of rehabilitation in more normal diurnal lighting conditions. Furthermore the mean minimum diameter of the optic nerve fibres appears to be permanently increased in the dark-reared rats. This increase appears to be due to a rise in the diameter of the optic nerve axons rather than to a change in the thickness of the myelin sheath.

The only previously published studies of the effects of dark-rearing during early life on the optic nerve of rodents were those published by Gyllensten & Malmfors (1963) and Gyllensten *et al.* (1966). In these early studies, rearing mice in complete darkness from birth for periods up to four months of age caused no significant changes in the optic nerve fibre composition compared with light-reared controls. However, Gyllensten *et al.* (1966) used phase contrast microscopy for the counting and measuring procedures. The resolution available with these light microscope techniques is much less than that possible with electron microscopy. It therefore seems probable that in the studies by Gyllensten *et al.* (1966) a large number of fibres was too small to be resolved and was therefore not counted. This possibility is borne out by comparing the estimates of total fibre number obtained by Gyllensten *et al.* (1966) with that of others in the literature. Thus Gyllensten *et al.* (1966) found that adult mice had about 50000 fibres per optic nerve. The values published more recently in the literature by researchers using high resolution electron microscopy for the counts on adult rat optic nerves have ranged from about 100000 to 140000 (Forrester & Peters, 1967; Hughes, 1977; Lam, Sefton & Bennett, 1982; Perry, Henderson & Linden, 1983; Bedi & Warren, 1983; Simpson & Bedi, 1984; Hunter & Bedi, 1986).

It is of some interest to speculate on the mechanisms involved and the possible functional significance, if any, of the observations made in the present study. It is possible that the increase in the diameter of the optic nerve axons during a period of decreased sensory activity may be due to alterations in the degree of storage of

neurotransmitters and/or related substances. Other possibilities include changes in the axonal plasma flow and terminal secretion brought about by the decreased activity. Whatever the precise mechanisms, support for the suggestion that decreased activity can result in an increase in the diameter of optic nerve axons has been provided by some recent observations made by Matheson & Roots (1988). These workers found that the optic nerve axons of goldfish kept at low temperatures had a larger diameter than those of fish kept at higher temperatures. It was suggested that the lower rates of conduction in the optic nerve fibres at the lower temperatures was partially compensated for by the increase in the diameter of the fibres of these fish.

It is known that larger diameter and myelinated optic nerve axons have conduction velocities which are generally greater than small diameter or non-myelinated axons (Fukuda, 1977). Given the slightly increased axonal diameters observed in the dark-reared rats it is a possibility that they may have somewhat greater conduction velocities in the optic nerve fibres than the light-reared control rats. If so, the exact benefits, if any, of this to the dark-reared animals is unknown. It appears that this aspect requires further research.

Several previous workers (e.g. Lam *et al.* 1982; Simpson & Bedi, 1984) have shown that the rat is born with many more optic nerve fibres than survive into adult life. The excess axons are removed during the first few days (Lam *et al.* 1982) or weeks of life (Simpson & Bedi, 1984). This process is accompanied by the loss of retinal ganglion cells (Potts, Dreher & Bennett, 1982). In the present experiment we have observed a small but significant drop in the number of optic nerve fibres between 30 and 65 days of age in both control and dark-reared rats, the actual values falling from about 140000 to between 110000 and 120000. Our present results therefore suggest that the decline in the number of optic nerve axons can continue into early postweaning life. This is contrary to the findings of Lam *et al.* (1982) but goes to support the findings of Simpson & Bedi (1984). This process of elimination does not seem to be significantly affected by dark-rearing during the first 30 postnatal days of life. This in turn suggests that ganglion cell and hence optic nerve axon elimination is a preprogrammed developmental event not easily influenced by environmental manipulations.

Bedi & Warren (1983) reported the possibility that the number of optic axons rises by a small but significant amount following this initial decline. Our present results do not support this finding. The fibre counts in the present study were carried out at a magnification of around 38000 whereas in the previous study (Bedi & Warren, 1982) the magnification used was of the order of 7000. It is therefore a possibility that Bedi & Warren (1982) may have missed counting some of the very small unmyelinated fibres in their study.

In conclusion, our experiments have shown that rearing rats in total darkness can affect the diameters of the optic nerve axons and the proportion of myelinated optic nerve fibres. These effects seem to be relatively long lasting, as a period of rehabilitation in normal lighting conditions did not restore some of these changes. However, dark-rearing appeared to have no significant effect on the total number of optic nerve fibres.

SUMMARY

Male rats were placed in complete darkness from birth until 30 days of age, followed in some cases by a 35 days period of rehabilitation in control lighting conditions. Groups of control and experimental animals were killed at 30 and 65 days of age by perfusion with buffered 2.5% glutaraldehyde. The right optic nerve was dissected out from each animal and processed for embedding in Epon. Quantitative

stereological procedures were used to estimate the total number of both myelinated and non-myelinated optic nerve fibres and their mean minimum diameters.

There were no significant differences in the total number of optic nerve fibres between dark- and light-reared rats. However dark-reared rats had myelinated and non-myelinated fibres with significantly larger fibre diameters than those in age-matched light-reared rats. The proportion of optic nerve fibres which were myelinated increased with age in both groups of animals. However by 65 days of age the degree of myelination was slightly but significantly greater in the previously dark-reared rats than in the light-reared controls. These results indicate that rats reared in complete darkness for the first 30 days of postnatal life show morphological changes in the optic nerves. The possible significance of these changes is discussed.

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